

DESCRIPTION

THIOREDOXIN REDUCTASE II

5 Technical Field

10 The present invention relates to a gene encoding a novel protein having a thioredoxin reductase activity and this protein itself. This protein is likely to closely relate to systems, for example, apoptosis, cancerization, or inflammation and expected to be widely applied to a research material for a therapeutic agent and a diagnostic marker.

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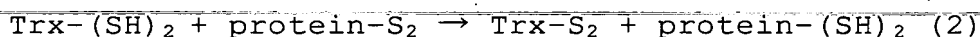
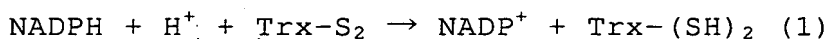
Background Art

15 It is known that viral infection causes apoptosis in cells in a host. This phenomenon is thought to be one of defense mechanisms for removing infected cells from a living body. Against this, viruses furnish an apoptosis inhibitory system for gaining time to proliferate themselves. Namely, an inhibitor of apoptosis protein (IAP) produced by viruses is one of anti-apoptosis proteins which inhibit apoptosis in a host. The presence of homologues for IAP was found in higher animals as well as in viruses. As a human IAP homologue, HIAP1, HIAP2 and XIAP (X-linked Inhibitor of apoptosis protein) have been reported. Among them, HIAP1 and HIAP 2 were clarified to bind to TRAF2 (TNFR associated factor 2) (Cell 83 (7): 1243-52. 1995., Proc Natl Acad Sci USA 94 (19): 10057-62. 1997). On the other hand, any factor binding to XIAP has not been identified yet. In order to analyze functions of XIAP involved in the inhibitory mechanisms of apoptosis in humans, its binding factor is necessary to be identified.

30 On the other hand, the following has been revealed about thioredoxin reductase (abbreviated to TxR, hereafter). Namely, TxR is involved in DNA transcription mechanism and cancer proliferation through the production of thioredoxin. The following is the reported knowledge.

35 Thioredoxin reductase; TxR (EC 1.6.4.5) is one of pyridine nucleotide-disulfideoxidoreductase families. This family includes

glutathion reductase, lipoamide dehydrogenase, tripanothion reductase, mercury ion reductase, and NADPH peroxidase. These proteins form a dimer, and have a disulfide bond at a redox active center. Flavin adenine dinucleotide (abbreviated to FAD) is used as co-enzyme to reduce a substrate using reduced form nicotina amide adenine dinucleotide phosphate (abbreviated to NADPH). Thioredoxin reductase oxidizes NADPH to  $\text{NADP}^+$  and converts oxidized form thioredoxin ( $-\text{S}_2$ ) which is a substrate to reduced form thioredoxin ( $-\text{SH})_2$  (1). Reduced form thioredoxin reduces a disulfide ( $\text{S-S}$ ) bond in a protein and becomes oxidized form thioredoxin itself (2). Thioredoxin is abbreviated to Trx hereafter.



Trx is a redox protein, and plays an important role as an electron donor which creates the reduced state in vivo. Trx is an electron donor to an enzyme, for example, ribonucleotide reductase, methionine sulfoxide reductase (Annu. Rev. Biochem 54: 237-71, 1985), vitamin K epoxide reductase (Biochem. Biophys. Res. Commun., 155 (3): 1248-54, 1988). Moreover, Trx catalyses a holding in a protein, and determines a DNA binding capacity of a transcription factor. The following substances are known as a transcription factor in which a DNA binding capacity is controlled by Trx.

NF- $\kappa$ B (J. Biol. Chem. 268 (15): 11380-8. 1993.) (Nucleic Acids Res. 20 (15): 3821-30. 1992)

TFIIIC

BZLF1 (Oncogene 6 (7): 1243-50. 1991.)

Glucocorticoid

p53

In addition, a transcription factor AP-1 is reduced by Ref-1 to have a DNA binding ability, and this Ref-1 is reduced through Trx.

On the other hand, TxR is getting attention as a target for an anticancer agent. For example, secretory type Trx has been reported to have a cytokine-like function and especially reduced form Trx has been reported to be essential for cell proliferation. It is TxR that produces reduced form Trx. Interestingly, in some kind of cancer, concentration of Trx in blood has been reported to increase and TxR

protein has also been reported to increase. It has been reported that insertion of mutation in the Trx redox active center and over expression thereof in oncocytes almost completely inhibited proliferation of oncocytes. From such a background, to terminate proliferation of oncocytes, recently inhibitors of TxR have aggressively been screened. Quinone and nitrosourea, which are anticancer agents, and retinoic acid, which terminates cell proliferation and is a differentiation-inducing agent, have the function of inhibiting TxR.

TxR is a protein containing Se (selenium) which is an essential trace element, as Secys (selenocysteine). Interestingly, Secys is the 21st amino acid which can be translated, and has a unique biosynthetic function by which Secys is encoded by the stop codon UGA. Secys has been also reported to have the radiation protective function and an anticancer effect. As a protein containing Secys, glutathione peroxidase (GPx) which reduces and deletes an active oxygen species hydroperoxide (-OOH), dependently on glutathione and Trx, and type I tetraiodothyronine deiodinase which converts thyroid hormone (thyroxine) precursor T4 into an active form T3, as well as selenoprotein P comprising 10 Secys and selenoprotein W, low molecular weight Secys-containing protein, present in muscles as the proteins which functions have not been well understood, have been reported. The previously reported human TxR has been reported to encode Secys by an amino acid sequence of Cys-Secys-Gly-stop codon (UAA). Absence of the activity of the most understood bovine TxR by treating with carboxypeptidase Y to remove Secys at C-terminus suggested that this C-terminus Secys is reported to be essential for the activity (Zhong, L., E. S. Arn-er, et al. (1998). Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. J. Biol Chem 273 (15): 8581-91.). Revealing the structure in a novel selenoprotein contributes studies in selenoproteins.

### 35 Disclosure of the Invention

An objective of the present invention is to isolate an

XIAP-binding protein and a DNA encoding the same. In addition, the present invention aims to isolate a novel protein having a TxR activity derived from human, and a DNA encoding the same.

The present inventors searched for an XIAP-binding protein using the yeast two hybrid system. As a result, a gene encoding an XIAP-binding protein has been successfully isolated from a human placenta cDNA library. A protein encoded by this gene was found to have a TxR activity to complete the present invention. Specifically, the present invention relates to the following proteins, DNAs encoding the same, methods for producing the same, and uses thereof.

(1) A protein comprising the amino acid sequence of SEQ ID NO: 2 or 4.

(2) A protein comprising the amino acid sequence of SEQ ID NO: 2 or 4 in which one or more amino acids are replaced, deleted, added, and/or inserted, having homology of 60% or higher to the amino acid sequence of SEQ ID NO: 2 or 4, and having a thioredoxin reductase activity.

(3) A protein having a thioredoxin reductase activity, encoded by a DNA which hybridizes to the DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3.

(4) A protein comprising the amino acid sequence of SEQ ID NO: 2 or 4 in which one or more amino acids are replaced, deleted, added, and/or inserted and having an XIAP-binding activity.

(5) A protein encoded by a DNA which hybridizes to the DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3, and having an XIAP-binding activity.

(6) An antibody binding to the protein of any one of (1) to (5).

(7) A cDNA encoding the protein of any one of (1) to (5).

(8) A cDNA comprising a protein coding region of the nucleotide sequence of SEQ ID NO: 1 or 3.

(9) A vector into which the DNA of (7) or (8) has been inserted.

(10) A transformant carrying the vector of (9).

(11) A method for producing the protein of any one of (1) to (5), the method comprising culturing the transformant of (10).

(12) An antisense DNA against all or a part of the cDNA of (7).

(13) An oligonucleotide comprising a strand of at least 15

nucleotides and hybridizing to the cDNA of (7).

(14) A DNA encoding a protein with a thioredoxin reductase activity and comprising the first exon or the second exon, and the third to the nineteenth exons below:

- 5 the first exon, SEQ ID NO: 18;  
 the second exon, SEQ ID NO: 19;  
 the third exon, SEQ ID NO: 20;  
 the forth exon, SEQ ID NO: 21;  
 the fifth exon, SEQ ID NO: 22;  
 10 the sixth exon, SEQ ID NO: 23;  
 the seventh exon, SEQ ID NO: 24;  
 the eighth exon, SEQ ID NO: 25;  
 the ninth exon, SEQ ID NO: 26;  
 the tenth exon, SEQ ID NO: 27;  
 15 the eleventh exon, SEQ ID NO: 28;  
 the twelfth exon, SEQ ID NO: 29;  
 the thirteenth exon, SEQ ID NO: 30;  
 the fourteenth exon, SEQ ID NO: 31;  
 the fifteenth exon, SEQ ID NO: 32;  
 20 the sixteenth exon, SEQ ID NO: 33;  
 the seventeenth exon, SEQ ID NO: 34;  
 the eighteenth exon, SEQ ID NO: 35; and  
 the nineteenth exon, SEQ ID NO: 36.

(15) The DNA of (14), described by SEQ ID NO: 37.

- 25 (16) A DNA hybridizing to the nucleotide sequence of any one of SEQ ID NOS: 18 to 36 or a part thereof, which can hybridize to human chromosome 22q11.2.

- (17) A DNA which can hybridize to all or a part of a portion of the nucleotide sequence of SEQ ID NO: 37, the portion non-overlapping  
 30 with the nucleotide sequences of SEQ ID NOS: 18 to 36.

(18) A method for screening a compound having an activity of inhibiting a binding of XIAP with the binding factor, the method comprising the steps of:

- (a) contacting simultaneously a candidate substance as a subject for  
 35 screening, and XIAP with the protein of (2), or

(a)' contacting a candidate substance as a subject for screening with

XIAP, and then, further contacting with the protein of (2),  
 (b) determining the amount of the protein of (2) which binds and/or  
 does not bind to XIAP, and  
 (c) selecting a compound which inhibits binding of XIAP with the  
 protein of (2).

(19) A method for screening a compound having an activity of promoting  
 or inhibiting an enzyme activity of thioredoxin reductase II, the  
 method comprising the steps of:

(a) contacting a candidate substance as a subject for screening with  
 the protein of any one of (1) to (3),  
 (b) observing the change in a thioredoxin reductase activity of the  
 protein of any one of (1) to (3), and  
 (c) selecting a compound which promotes or inhibits an enzyme  
 activity of thioredoxin reductase II.

SEQ ID NOs: 2 and 4 show amino acid sequences for a novel protein  
 TxRII $\alpha$  and protein TxRII $\beta$ , respectively, which have been obtained  
 by the present inventors, and SEQ ID NOs: 1 and 3, respectively, show  
 nucleotide sequences of cDNA encoding the same. In the following  
 specification, TxRIIs is used as a term simultaneously containing  
 both TxRII $\alpha$  and TxRII $\beta$ . These amino acid sequences were deduced  
 based on novel genes structures of which were determined by screening  
 based on a human placenta cDNA library by applying the two hybrid  
 system. The two hybrid method is for confirming interaction among  
 proteins with high sensitivity. The principle is the method for  
 screening a combination of interacting proteins using the expression  
 of a marker gene as an index, as described in Examples. The present  
 inventors applied this method for searching for a substance having  
 an avidity to XIAP to discover a novel factor and reveal the  
 structure.

A location of a gene encoding TxRIIs provided by the present  
 invention was confirmed to be 22q11.2 on chromosomes. Both TxRIIs  
 are present in 70 kbp in this region, by separating into 19 exons.  
 The genes were mapped on chromosomes by database searching, and the  
 presence of genes for proteins having TxR activity in this location  
 was not known at all. TxRII $\alpha$  and TxRII $\beta$  were determined to be  
 alternative splicing forms of TxRII because they comprised the

identical structure in the second and the following exons. Specifically, the first exon of TxRII $\alpha$  is Exon 1 below (SEQ ID NO: 18), and the first exon of TxRII $\beta$  is Exon 2 below (SEQ ID NO: 19). The second and the following exons of the both, from Exon 3 (SEQ ID NO: 20) to Exon 19 (SEQ ID NO: 36), are identical.

Interestingly, causative genes of, for example, Di George syndrome, and neurofibromatosis, are mapped close to these TxRII genes, and the possibility of involvement of TxRIIs discovered by us in some inherited disease can not be denied. More importantly, the exon 1 of TxRII $\alpha$  is overlapped with a promoter region of catechol-o-methyltransferase (EC 2.1.1.6, abbreviated to COMT, hereafter). COMT was also mapped at 22q11.1 11.2 on chromosomes and the direction of transcription was reverse against the TxR II. Namely, it was suggested that, when transcribed, TxR II $\alpha$  possibly inhibited the expression of COMT by acting on mRNA of COMT in an antisense manner. This may be a cause of schizophrenia and Parkinson's disease. These facts suggest that transcription of COMT can be efficiently inhibited by overexpressing the sense strand DNA of exon 1 in TxRII $\alpha$  or administering an antisense oligonucleotide or a sense nucleic acid analogue.

Information on the locations of the exons and introns in the genomic DNA provided by the present invention is essential for studying the relationship between these diseases and genetic abnormalities, and may provide a probe for diagnosing these genetic abnormality. Table 1 shows the location for each exon in genome. Each number indicating a location described below is the number when 5' end of the genomic nucleotide sequence in SEQ ID NO: 37 is 1. Based on these information, for example, a DNA hybridizing specifically to each exon can be used as a primer for amplifying an intron part. In contrast, a DNA hybridizing to an intron region except for each exon in the nucleotide sequences of SEQ ID NO: 37 can be used for amplifying an exon by PCR. These primers are essential tools for detecting abnormality in exons and introns. Because inherited diseases may result not only from abnormality in a protein coding region, but also from the abnormality in an intron, leading to the event in which splicing does not occur correctly. Therefore, these

kinds of primers are useful for revealing the inherited diseases. In addition, a DNA which can hybridize to an exon is useful as a probe. Especially, a DNA specifically hybridizing to chromosome 22q11.2 among these DNA is useful as a probe for cloning the genomic DNA of SEQ ID NO: 37 by the present invention. Specifically, by screening a human genomic library as a source with these probes, the genomic DNA of SEQ ID NO: 37 can be isolated. In the case of using as a probe or a primer, the oligonucleotide based on the present invention comprises at least 15 nucleotides to achieve hybridization under stringent conditions, preferably of 15 to 200 nucleotides, and more preferably of 25 to 100 nucleotides.

Table 1

		The structure of a splicing part		SEQ ID NO:
Exon	Nucleotide No.	3 side	5' side	
Exon 1	1-103	agcag/GTA		18
Exon 2	9247-9446	ccaag/GTG	CAG/caggtc	19
Exon 3	10706-10774	ggagg/GTA	CAG/ccgccc	20
Exon 4	22205-22261	ccaag/GTA	CAG/gcacc	21
Exon 5	22800-22944	gactg/GTA	CAG/gagga	22
Exon 6	23587-23661	gacag/GTA	CAG/aaaag	23
Exon 7	25961-26039	aagag/GTG	CAG/attct	24
Exon 8	26529-26591	cgcac/GTG	CAG/atcga	25
Exon 9	30358-30428	aaaac/GTA	CAG/gttgg	26
Exon 10	43016-43035	cagct/GTA	CAG/atgtg	27
Exon 11	43954-44045	accag/GTA	CAG/caaat	28
Exon 12	46503-46677	catag/GTA	CAG/gtcga	29
Exon 13	58623-58759	tggag/GTA	AAG/gggcg	30
Exon 14	61367-61462	acaat/GTG	CAG/gttct	31
Exon 15	61813-61905	ttgag/GTG	CAG/gtcta	32
Exon 16	63647-63718	taaag/GTG	CAG/atggt	33
Exon 17	63897-63994	atcaa/GTA	CAG/gtgtg	34
Exon 18	64850-65044	cccag/GTA	CAG/gatgg	35
Exon 19	66277-66566	-		36



The amino acid sequence of SEQ ID NO: 2 showed 55% homology to the known human TxR by searching database, and 38% homology to human glutathione reductase. Especially, in a redox active center, a FAD-binding region, a NADPH-binding region, and a selenocysteine active center, homology was completely conserved. Figure 1 shows alignment of amino acid sequences for TxRII $\alpha$  and the known TxR. The present inventors named the protein comprising the amino acid sequence of SEQ ID NO: 2 TxRII $\alpha$ , based on these data. Because an avidity with XIAP is not seen in the known human TxR, the protein of the present invention is novel. Homology between these two amino acid sequences does not reach 60%. Therefore, these two are different proteins, and human TxR does not predict structures and functions of TxRII $\alpha$  or TxRII $\beta$ .

It has been reported that human thioredoxin reductase reported in 1995 contains a sequence of AUUUA in the untranslated region present at 3' side (abbreviated to 3' UTR hereafter). This AUUUA is considered to contribute instability of mRNA and it has been reported that mRNA is rapidly decomposed by the presence of this sequence in 3' UTR. This kind of sequences has been also reported in cytokines and protooncogenes, and it has been known that these proteins increase at once by a stimulus and disappear. These facts suggest that the previously reported human thioredoxin reductase is transiently transcribed and translated by some stimulus and decomposed immediately after that, and that, thus, the effect is limited to a very temporary one. In contrast, this kind of sequences is not present in 3' UTR of TxRIIs of the present invention, and TxRIIs are considered to be constantly involved in controlling redox *in vivo*. Therefore, inhibitors and promoters for TxRIIs are likely to be completely different from the reported inhibitors of TxR in terms of specificity, inhibitory effects, and as therapeutic agents. Therefore, the knowledge regarding TxRIIs, revealed by the present invention, has an important meaning in the development of drugs involved in redox control *in vivo*.

The proteins of the present invention contain not only those disclosed in SEQ ID NOs: 2 and 4, but also mutants having the physiological activity at the same level. Specifically, the present

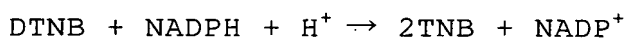
invention contains the protein comprising the amino acid sequence of SEQ ID NO: 2 or 4, or a protein having an XIAP-binding activity and comprising the amino acid sequence in which one or more amino acids are replaced, deleted, added, and/or inserted. Alternatively  
5 the proteins of the present invention contain the protein comprising the amino acid sequence of SEQ ID NO: 2 or 4, or all proteins comprising the amino acid sequences in which one or more amino acids are replaced, deleted, added and/or inserted, and desirably having 60% or higher homology as a whole to the above amino acid sequence  
10 and a TxR activity.

As understood from the amino acid sequence of SEQ ID NO: 2 or 4, TxRIIs of the present invention are a selenoprotein containing selenocysteine in a molecule. On the other hand, the previously  
15 reported human TxR has been reported to encode Secys by an amino sequence of Cys-Secys-Gly-stop codon (UAA). Moreover, in bovine TxR, this Cys-Secys-Gly at C terminus is a region essential for the activity expression of TxR. Therefore, in human TxRIIs by the present invention, this region is considered to have an important meaning in the TxR activity expression.

A method for adding mutation in an amino acid sequence while maintaining a physiological activity is known. For example, as a method for preparing a mutant using the random mutation, the chemical mutagenesis method (Myers RM, et al. Methods Enzymol., 1987; 155: 501-527) is known. In this method, a random mutation is introduced  
20 into a single-stranded DNA of a target gene by adding a nucleotide modification reagent. Then, a double-stranded DNA is synthesized by using appropriate primers with the obtained single stand DNA as a template by PCR and cloned. A target mutant can be obtained by selecting a clone which provides an expression product having an desired activity from a library of mutants. On the other hand, as  
25 a method for preparing a mutant by determining a target nucleotide, a method for introducing the mutation by conducting PCR with a target gene as a template using mutation oligonucleotide primers is known (Ito W. et al., Gene 1991 June 15; 102 (1): 67-70). Mutation in an  
30 amino acid sequence occurs not only by an artificial manipulation but also in the natural condition. A mutant of the present invention  
35

includes such a naturally occurring mutant as long as it maintains the TxR activity or the XIAP-binding activity.

As a method for confirming the TxR activity, the following two methods are known for example (Holmgren, A. et al. Methods Enzymol. 252: 199). First, using an appropriate SH indicator, such as 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), 5-mercapto-2-nitrobenzoic acid (TNB) produced by the TxR activity is measured with absorbance at 412 nm by a thiol. This reaction is shown as follows:



The other index for the TxR activity is a method called an insulin assay in which an enzyme activity is monitored by tracing a change created by the cleavage of the SS-bond of insulin by reduced form Trx resulted from the TxR activity. As a change which is an index, the decrease of absorbance at 340 nm by oxidation of co-enzyme NADPH, and absorbance at 412 nm of a thiol group resulted from reduction of insulin are used. Production process of reduced form Trx by TxR is as described above.

On the other hand, an XIAP-binding activity functionally equivalent to the binding activity in the natural form TxRII $\alpha$  or  $\beta$  comprising the amino acid sequence of SEQ ID NO: 2 or 4 can be used. As a method for screening functionally equivalent substances, specifically, for example, the following methods can be used. Specifically, the method is a method for screening a compound having an activity of inhibiting the binding of XIAP with the binding substance and comprising the following processes (a) or (a)', (b), and (c):

(a) contacting simultaneously a candidate substance as a subject for screening, and XIAP with the protein of the present invention, or (a)' contacting a candidate substance as a subject for screening with XIAP, and then, further contacting with the protein of the present invention,

(b) determining the amount of the protein of the present invention which binds and/or does not bind to XIAP, and

(c) selecting a compound which inhibits binding of XIAP and the protein of the present invention.

More specifically, a method according to the method shown as an inhibitor assay of Example 7-5) can be presented. If a diluted series of a candidate compound is used as a sample and the decreased absorbance is observed dependently on the diluted series, the candidate compound is judged to have a binding inhibitory activity. Alternatively, a combinatorial chemistry can be applied. Specifically, a library of candidate compounds is prepared, and the proteins of the present invention are added thereto with XIAP to monitor XIAP to be bound to the candidate compound to screen an antagonistic inhibitory substance for TxRIIs. On the other hand, a compound which blocks the binding of TxRIIs to XIAP can be screened by using TxRIIs which bind to a candidate compound as an index.

In the screening method by the present invention, any proteins can be used as the above-described protein of the present invention as long as it comprises a binding activity domain with XIAP. Specifically, a protein is not necessarily the complete molecule of the amino acid sequence of SEQ ID NO: 2 or 4. In order to observe a binding of candidate compound or the protein of the present invention, these proteins are modified with an observable molecule. As an observable molecule, for example, radioactive isotopes, fluorescent substances, luminescent substances, and enzymatic active substances can be used. In the case of applying the above combinatorial chemistry, an immobilized library of candidate compounds on a solid phase is useful as isolation of reaction solution, washing, and the following measurement of labels are easily manipulated.

These methods can be used, not only for screening mutants in the present invention, but also, for screening compounds which inhibits the binding of XIAP and the protein of the present invention in general. Because a compound screened by this method can control signal transduction system in which XIAP is involved, the proteins provided by the present invention, an antibody thereof, an analogue thereof and such can be expected to have effects of inhibiting cancer, inducing cell death in virus infected cells through promotion of apoptosis, etc.

In addition, a method for screening a compound having an

activity of promoting or inhibiting the enzyme activity can be provided by using TxRIIs of the present invention. This method comprises the steps of:

- (a) contacting a candidate substance as a subject for screening with TxRIIs,
- (b) observing the change in the TxR activity of TxRIIs, and
- (c) selecting a compound which promotes or inhibits the TxR activity in TxRIIs.

TxRIIs to be used for this screening are not necessarily a complete molecule, and a fragment maintaining an enzyme activity of TxRIIs can be used. The TxR activity can be measured based on a method such as the above method. Because the structure of TxRIIs is different from the known TxRI, a compound which affects one activity does not necessarily affect the other. Therefore, a method for screening a substance which affects an enzyme activity of TxRIIs is an essential technique for identifying inhibitors and activators specific to the enzyme activity of TxRIIs or obtaining a compound which affects TxRI in the same manner as in TxRIIs.

As TxR controls redox *in vivo*, an inhibitor for TxRIIs which can be obtained based on the screening method by the present invention can be expected to be used as an anticancer drug, or a therapeutic agent for autoimmune disorders. For example, an organic gold compound used as a general therapeutic agent for rheumatism, an autoimmune disorder, is considered to have a high inhibitory activity on selenoproteins, especially on TxR. Thus, a compound having an inhibitory effect on TxRII can be expected to have a similar pharmacological activity (Stephan Gromer et al., J. Biol. Chem. Vol. 273, No. 32, 20096-20101, 1998). Moreover, if a pharmacological activity through the inhibition of TxR activity is expected, the method for screening a compound which affects an activity of TxRII, provided by the present invention, is useful because it is necessary to select a compound effective not only to a known TxR but also TxRII.

The proteins of the present invention can be obtained by extracting and purifying from cells expressing TxRII $\alpha$  or  $\beta$ . Selecting cells which highly express a target protein is advantageous. Because the nucleotide sequence of DNA encoding the target protein

is provided, the method for screening cell lines which highly express the target gene by using a probe based on this sequence is routinely conducted by a person skilled in the art. As shown in Examples, TxRIIs by the present invention are expressed in many cultured cells, these cultured cells can be used as a material. A method for purifying a target protein by combining various extraction methods and protein purification methods from cell culture can be selected by a person skilled in the art from experiences. Specifically, various purification methods, for example, gel filtration, ion exchange chromatography, reversed phase chromatography, immuno affinity chromatography, can be used.

Apart from the purification from these natural materials, the proteins of the present invention can be obtained by the genetic engineering technique. For example, an expression vector is constructed by inserting a translation region to an appropriate vector based on the nucleotide sequence of SEQ ID NO: 1 or 3. Then this expression vector is transfected to an appropriate host to express the target TxRII as a recombinant.

In addition, the present invention provides cDNAs encoding the above proteins of the invention. The DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 3 disclosed in the present invention is novel. Regarding cDNA of the present invention, the target gene can be obtained by screening a cDNA library using a probe designed based on, for example, the nucleotide sequence of SEQ ID NO: 1 or 3. Alternatively, the gene of the present invention can be obtained by synthesizing primers based on the nucleotide sequence of SEQ ID NO: 1 or 3, and conducting PCR using a cDNA library as a template. Probes and primers can be designed and prepared based on the nucleotide sequences of cDNA of the present invention by the methods known to a person skilled in the art. In primers for PCR, sequences close to 5' end and 3' end of a fragment to be amplified are selected. Addition of a restriction enzyme recognition site to 5' side of the primers is convenient for insertion into a vector. Both nucleotide sequences of SEQ ID NOs: 1 and 3 comprise the length of about 2 kbp. A whole region of such a length can be amplified by conducting PCR once using a pair of primers and cDNA as a template. A target gene

can be sensitively detected by confirming an amplification product to be obtained by electrophoresis. An expression vector can be constructed by inserting the amplification product into a vector. A commercially available library used in Examples contains a full length cDNA of TxRIIs by the present invention. Therefore, by conducting PCR using this as a template, the cDNA of the present invention can be easily obtained. Alternatively the cDNA of the present invention can be obtained by conducting RT-PCR based on mRNA in each cell line which shown the expression of TxRIIs. An element of 3'UTR is as important as CDS at the construction of the expression vector for active form of TxRIIs, based on the nucleotide sequences of SEQ ID NO: 1 or 3. Among 3' UTR, the portions corresponding to 1780-1909 of SEQ ID NO: 1 and 1883-2012 of SEQ ID NO: 3 (SEQ ID NO: 5, 130 bp) constitute a common nucleotide sequence. This part is essential for expressing a complete form of TxRIIs containing selenocysteine. UGA which is a stop codon in general, is translated to selenocysteine by the stem loop structure composed of a part corresponding to this region in mRNA. As previously described, selenocysteine is considered to be an amino acid essential for an enzyme activity of TxR. Therefore, into the expression vector of the present invention, an insert should be inserted in the form containing this region. If the TxR activity is not expected to TxRIIs of the present invention, this region is not essential. For example, in the case of aiming the expression of a domain peptide of TxRIIs composed only of a specific region, a target protein can be obtained by inserting only the nucleotide sequence encoding a necessary amino acid sequence in the form able to express. To the domain peptide obtained in this manner, an enzyme activity of TxR can not be expected. However, for example, the domain peptide can be used as an immunogen for preparing an antibody which recognizes TxRIIs by the present invention. Alternatively, a mutant with a binding-activity with XIAP based on the present invention can be prepared by selecting a region serving the biding with XIAP.

The DNA of the present invention contains not only the DNA constituted by the nucleotide sequence of SEQ ID NOs: 1 and 3, but also mutants thereof. Mutants of the DNA based on the present

invention are mainly classified to the following two. Specifically, first, a DNA comprising a nucleotide sequence encoding all proteins comprising mutation in the above amino acid sequence by the present invention is the DNA mutant based on the present invention. More specifically, a DNA encoding all mutants comprising mutation in the amino acid sequence within the range of maintaining an activity as TxRIIs are contained in the DNA of the present invention, regardless of being able to hybridizing to SEQ ID NO: 1 or 3 or not. Because several sequences correspond to codons for one amino acid in general (degeneracy), theoretically an astronomical number can be expected for a nucleotide sequences of DNA encoding a given amino acid sequence. From this reason, the DNA nucleotide sequences of the present invention must be identified regardless of complementarity to a specific sequence.

Second, a DNA which can hybridize to SEQ ID NO: 1 or 3, and encodes a protein having an activity as TxRIIs is included in the DNA of the present invention. Many of sequences which can hybridize to a specific sequence under stringent conditions are thought to have an activity similar to the protein encoded by the specific sequence. A specific example of hybridization conditions is 5xSSC, at 25°C in the absence of formamide, preferably, 6xSSC, at 25°C with 40% formamide, and more preferably, 5xSSC, at 40°C with 50% formamide. An example of washing after hybridization is 2xSSC at 37°C, preferably 1xSSC at 55°C, and more preferably 1xSSC at 60°C.

The nucleotide sequence of DNA of the present invention including mutants can be used for various uses based on the known technologies. Based on the cDNA nucleotide sequence identified in the present invention, an oligonucleotide which specifically hybridizes to this nucleotide sequence can be obtained. An oligonucleotide of the present invention is composed of at least 15 nucleotides in order to archive hybridization under stringent conditions, preferably of 15-200 nucleotides, and more preferably 25-100 nucleotides. Such a nucleotide can be used as a probe and a primer. Based on a given sequence, a person skilled in the art routinely designs a probe specifically hybridizing to the sequence. A nucleotide sequence archiving a specific hybridization is not



necessarily completely complementary on a target nucleotide sequence. Variation of sequences is acceptable as long as it can archive the necessary specificity under stringent conditions. An oligonucleotide comprising a determined nucleotide sequence can be obtained by the chemical synthesis. The oligonucleotide can be used for hybridization assays in various formats by adding an appropriate label to the oligonucleotide. In the case of using as a primer, multiple regions can be set depending on a synthesis principle for a complementary strand. For example, as a primer for PCR, a region determining both 5' and 3' sides in the segment which is an object of the synthesis is selected. The oligonucleotide of the present invention can be applied to various complementary strand synthesis reaction, for example, not only basic PCR, but also RT-PCR with RNA as a template, nested PCR which enables a sensitive detection by nesting a amplification region, cDNA synthesis, etc.

For example, as a primer for amplifying cDNA of TxRIIs, or for amplifying 3'UTR, the following nucleotide sequences can be presented. By using a primer for amplifying cDNA of TxRIIs described below, TxRIIs of the present invention can be distinguished from a known TxR and cDNA of the latter can be specifically amplified.

Forward primer for TxRII $\alpha$  (SEQ ID NO: 13):

5'-ACGATGGCGGCAATGGCGGTG-3'

Forward primer for TxRII $\beta$  (SEQ ID NO: 14):

5'-ACCATGGAGGACCAAGCAGGT-3'

Reverse primer for TxRIIs (SEQ ID NO: 15):

5'-TTACCCTCAGCAGCCTGTCAC-3'

Forward primer for 3'UTR (SEQ ID NO: 16):

5'-GCGCCATCCCTGCAGGCCAGG-3'

Reverse primer for 3'UTR (SEQ ID NO: 17):

5'-CACACTTCAGAAAAAGTACCC-3'

The oligonucleotide based on the present invention can be used as an antisense DNA which inhibits the expression of TxRIIs. There are more than one factors as an inhibitory effect of an antisense nucleic acid on the expression of a target gene (Hirashima and Inoue: "Shin-seikagaku Jikken Koza (New Biochemistry Experiment) 2 Nucleic Acid IV Replication and Expression of a gene", Edited by Japanese

Biochemistry Society, Tokyo-Kagakudojin, pp. 319-347, 1993). The expression of a target gene can be inhibited by any of the effects. In one embodiment, the translation of the gene is effectively inhibited by designing an antisense sequence complementary to non-translation region close to 5' end of mRNA in the gene. A sequence complementary to a coding region or a non-translation region at 3' side, however, can be used. A DNA including an antisense sequence of not only a translation region of a gene but also a non-translation region is included in the antisense DNA used in the present invention. An antisense DNA to be used is ligated downstream of an appropriate promoter and preferably a sequence containing a transcription termination signal is ligated to 3' side thereof. The DNA prepared in this manner can be transfected into cells in which the expression should be inhibited by a known method. A sequence of an antisense DNA is preferably complementary to an endogenous TxRIIs gene contained in cells to be transformed (or a homologous gene) or a part thereof, but is not necessarily completely complementary as long as it is able to effectively inhibit the expression of the gene. A transcribed RNA has preferably 90% complementarity, and the most preferably 95% complementarity on the transcription product of a target gene. To effectively inhibit the expression of the target gene using an antisense sequence, the length of an antisense DNA is at least 15 or more nucleotides, preferably 100 or more nucleotides, and more preferably 500 or more nucleotides. Ordinarily, the length of an antisense RNA to be used is shorter than 5 kb, and preferably shorter than 2.5 kb. The expression of an endogenous gene can be inhibited by using a DNA encoding a ribozyme.

The present invention provides an antibody which recognizes the protein based on the present invention. An antibody of the present invention can be prepared by immunizing the protein obtained in the above manner or a fragment thereof through a known method. In immunization, adjuvant, such as FCA, is mixed with an immunogen and subcutaneously immunized to an animal to be immunized by an appropriate immunization schedule. High immune stimulation can be expected by selecting an animal to be immunized, in which the structure of TxR is as different from that of human as possible. An

antibody can be prepared not only as a polyclonal antibody purified from serum of the immunized animal, but also as a monoclonal antibody which can be obtained by cloning antibody-producing cells. The method for collecting antibody-producing cells of an immunized animal and establishing cell lines which produce monoclonal antibodies by fusing the cell lines with cultured cell lines enabling cloning is obvious to a person skilled in the art. The antibody obtained in this manner can be used for immunologically detecting and purifying TxR by the present invention.

Moreover a gene in variable region of an antibody contained in antibody-producing cells which recognizes TxRIIs derived from animals of different species can be used for humanization. Specifically, for example, a chimeric antibody which comprises a constant region of a human antibody in the antibody variable region of a mouse can be created by gene recombination. A method for obtaining a so-called humanized antibody in which a hypervariable region is solely inserted into a framework of a human antibody is known. These humanized antibodies can be safely and effectively used *in vivo* because an immunological reaction is difficult to occur in the case of administering to human.

#### **Brief Description of the Drawings**

Figure 1 shows the alignment of amino acid sequences for TxRII $\alpha$  of the present invention and the known TxR.

Figure 2 is a photograph showing the result of detecting TxRIIs in each cultured cell line by Western blot method using an antiserum of mouse anti-TxRII $\alpha$ .

Figure 3 shows the TxR activity measured by the DTNB assay in the TxRII $\alpha$  recombinants fused with each tag. The vertical and horizontal axes indicate absorbance at 412 nm and reaction time, respectively.

Figure 4 shows the TxR activity measured by insulin assay in the TxRII $\alpha$  recombinants fused with each tag. The vertical and horizontal axes indicate change of absorbance at 340 nm and reaction time, respectively.

Figure 5 shows effects of the TxR activity inhibitor on the TxR

activity of the flag-tag fused TxRII $\alpha$  protein, measured by the DTNB assay. As a TxR activity inhibitor; 1-chloro-2, 4-dinitrobenzene (CDNB) and 13-cis-retinoic acid are used. The vertical and horizontal axes indicate absorbance at 412 nm and reaction time, respectively.

### Best Mode for Carrying Out the Invention

The present invention is illustrated in detail below based on the Examples.

All techniques used in the present invention followed J. Sambrook, E. F. Fritsch & T. Maniatis (1989) Molecular Cloning, a laboratory manual, second edition, Cold Spring Harbor Laboratory Press.

#### 1. Cloning of XIAP by PCR

##### 1-1) Preparation of primers

The following two primers were synthesized to isolate the full length human XIAP gene by PCR.

- 5' primer (XIAP2486 (32mer))

5'-GCG GGA TCC ATG ACT TTT AAC AGT TTT GAA GG-3'

\* 3 bases (GCG) at 5' end are for conveniently conducting the restriction enzyme treatment.

(GGATCC) from the 4<sup>th</sup> to the 9<sup>th</sup> bases at 5' end is a restriction enzyme BamH I site.

- 3' primer (XIAP 2482 (32 mer))

5'-GCG CTC GAG CTA CTA TAG AGT TAG ATT AAG AC-3'

\* 3 bases (GCG) at 5' end are for conveniently conducting the restriction enzyme treatment.

(CTCGAG) from the 4<sup>th</sup> base to the 9<sup>th</sup> base at 5' end is a restriction enzyme Xho I site.

##### 1-2) PCR

Using the cDNA derived from human T-cell-derived Jurkat cells as a template DNA, the full length human XIAP gene was amplified by PCR.

PCR was conducted with GeneAmp PCR System 2400 (PERKINELMER) by the following program.

- a) 94°C for 5 min
- b) 1 cycle of 94°C for 1 min, 58°C for 3 min, 72°C for 3 min
- c) 35 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 2 min
- d) 72°C for 10 min

5

### 1-3) Cloning of a PCR product to pAS2-1 vector

#### i) Purification of a PCR product

The amplified DNA fragment was confirmed by the 1% agarose electrophoresis after PCR. This DNA fragment was treated with restriction enzymes BamH I and Xho I. The DNA fragment treated with restriction enzymes was electrophoresed by the 1% agarose, excised and purified by Glass Matrix method (GeneClean, BIO101).

#### ii) Preparation of vector

Vector pAS2-1 is a bait vector used in MATCHMAKER Two Hybrid System (a product name) of Clontech, and comprises a multicloning site (MCS) downstream of a sequence encoding GAL4-DNA-BD (a DNA binding domain of GAL4 protein). A bait in the two hybrid system means a vector in the side which expresses a known protein functioning as a probe for searching unknown binding factors. To match translation frames for GAL4-DNA-BD and the PCR product, this MCS was digested with Nde I at the restriction enzyme Nde I site, blunt-ended by the standard method, and self-ligated to obtain the vector pAS $\Delta$ NdeI (+2) in which two frames were slipped. The fragment treated with restriction enzymes BamH I and Sal I was purified in the same manner for a PCR product. The purified product and the fragment of vector pAS2-1 $\Delta$ NdeI were ligated. The purified PCR product and pAS2-1 $\Delta$ NdeI were mixed in the molar ratio of 1, and reacted for 1 hour at 16°C with T4 DNA ligase.

#### iii) Transformation of *E. coli*

A ligation reaction solution was added to *E. coli* strain DH5 $\alpha$  made competent by the standard method (Hanahan, D. 1983 Studies on transformation of *Escherichia coli* with plasmids, J. Mol. Biol 166: 557), gently mixed, kept on ice for 30 min, heat-shocked for 90 sec in warm water at 42°C, kept on ice for 2 min again, and cultured with

shaking at 37°C for 1 hour in SOC medium. The product was spread on a LB plate containing 50 µg/ml ampicillin and cultured overnight at 37°C.

- 5 iv) Collection of DNA by the alkaline-SDS method and confirmation of an insert

Colonies were harvested from the plates and cultured in a LB-ampicillin medium at 37°C overnight. From the cultured *E. coli*, plasmid DNA was collected using the alkaline-SDS method. The collected plasmid DNA was cleaved by an appropriate restriction enzyme and insertion of the target PCR product into the vector was confirmed by the agarose electrophoresis.

- v) Confirmation of sequences

The collected DNA was purified by the polyethylene glycol precipitation method and the PCR product in the vector was confirmed by the fluorescence sequencer (PERKINELMER) based on the Sanger method. In this manner, plasmid DNA of pASΔNdeI (+2) -XIAP in which the full length human XIAP gene was inserted into pASΔNdeI (+2) vector was obtained.

## 2. 2 Hybrid screening

In analyses of intracellular information transduction mechanisms and studies on cellular mechanisms at higher levels, detection of interaction between proteins and identification of known or unknown molecules interacting with a known protein are very important. The two hybrid screening system has been given attention for detecting a interaction between proteins encoded by two genes, or as a method for cloning a molecule interacting a gene product. In this method, each of two gene products is fused to a DNA binding site (GAL4-DNA-BD) and a transcription activation site (GAL4-AD) in a transcription factor, to detect the interaction between two, using a transcription activity as an index. A GAL4-DNA-BD fusion protein and a GAL4-AD fusion protein are simultaneously expressed in a yeast nucleus. When the both interact, HIS3 gene comprising GAL4 promoter upstream and lac Z gene are expected to be transcribed and translated.

Specifically, the yeast can grow in the agar medium without histidine only in the presence of the interaction between the both, and  $\beta$ -galactosidase (abbreviated to  $\beta$ -Gal, hereafter) activity can be detected using X-gal as a substrate. The two hybrid screening system is so advantageous that interaction of two gene products can be judged in a yeast nucleus *in vivo* without purifying the proteins. However, a protein transcribed from the GAL4 promoter without showing interaction can not be screened. Therefore, it is very important to confirm that lac Z gene does not express only with the GAL4-DNA-BD fusion protein, namely, there is no  $\beta$ -Gal activity. For the two hybrid screening, the MATCHMAKER two hybrid system method 2 of CLONTECH was used and all experimental methods followed this protocol.

#### 2-1) Purification of a library DNA for pray

Human Placenta MATCHMAKER cDNA Library purchased from CLONTECH was used as a library for screening. This library was prepared by pACT2 vector and contains a MCS downstream of a sequence encoding GAL4-AD (an Activation Domain of the GAL4 protein) and a cDNA fragment was inserted into this MCS. In the two hybrid system method, a library predicted to contain unknown binding factors is called a pray. About 20,000 colonies per an LB-ampicillin plate with a diameter of 150 mm were spread and these 100 plates were cultured at 30°C overnight and bacterial cells were cultured in a LB ampicillin liquid medium at 30°C for 4 hours. Plasmid DNA was collected from the harvested *E. coli* cells by the polyethylene glycol precipitation method and purified.

#### 2-2) Confirmation of expression of a fusion protein and the absence of $\beta$ -Gal activity

Yeast was transformed by the constructed pAS $\Delta$ NdeI (2+)-XIAP, and expression of XIAP as the GAL4-DNA-BA fusion protein, and an activation of GAL4 promoter solely by the GAL4-DNA-BD fused XIAP (bait) but no-expression of lac Z gene were confirmed.

Yeast Y 190 made competent by the lithium acetate method (Gietz, D., Jean A., Woods, R. A., & Schiestl, R. H. 1992, Improved method

for high efficiency transformation of intact yeast cells. Nucleic Acid Res. 20: 1425) was transformed by using plasmid DNA of pAS $\Delta$ NdeI (+2)-XIAP. Colonies obtained by transformation were cultured in the SD/-Trp liquid medium at 30°C for 3 days. After the culture, yeast cells were harvested by centrifugation, and proteins were extracted from yeast by the standard method (Printen, J. A. & Sprague, G. F., Jr. (1994) Protein interactions in the yeast pheromone response pathway: Step 5 interacts with all members of the MAP kinase cascade. Genetics 138: 609-619), using the urea/SDS protein extraction buffer. After electrophoresis of proteins by SDS-PAGE, the proteins were blotted on the PVDF protein. The expression of the fusion protein of GAL4-DNA-BD and XIAP was confirmed by Western blot using the anti-GAL4 DNA binding domain monoclonal antibody (CLONTECH) and anti-XIAP polyclonal antibody.

A sterile nylon transfer membrane (Hybond-N+, Amersham) was placed on the plate on which yeast transformants in which expression of the fusion protein between GAL4-DNA-BD and XIAP was confirmed were grown. Thus, the colonies were transferred to the membrane. This membrane was immersed in liquid nitrogen for 10 sec, returned to room temperature, placed on a filter paper immersed with the Z-buffer/X-gal solution (100 ml Z-buffer (16.1 g/L Na<sub>2</sub>HPO<sub>4</sub>-7H<sub>2</sub>O, 5.50 g/L NaH<sub>2</sub>PO<sub>4</sub>-H<sub>2</sub>O, 0.75 g/L KCl, 0.246 g/L MgSO<sub>4</sub>-7H<sub>2</sub>O, adjusted to pH 7.0), 0.27 ml mercaptoethanol, 1.67 ml X-gal solution (20 mg/ml X-gal in DMFA)) with the surface with colonies up, and kept at 30°C for one hour or longer. As a result, the yeast transformants in which the expression of the fusion protein of GAL4-DNA-BD and XIAP was confirmed did not turn blue. Specifically, sole the fusion protein of GAL4-DNA-BD and XIAP did not activate transcription from the GAL4-promoter, confirming that the two hybrid screening system can be used.

### 2-3) The primary screening

The yeast transformants in which the expression of the fusion protein of GAL4-DNA-BD and XIAP was confirmed were mass-cultured and were made competent by the lithium acetate method. These were transformed by the previously prepared Human Placenta MATCHMAKER



cDNA library. The obtained transformants were streaked on the plates of SD/-Trp/-Leu/-His/+3-AT, and cultured for 7 days at 30°C. By this, only yeast in which bait bound to pray and His3 gene downstream of the GAL4 promoter expressed to become His<sup>+</sup> can only grow to form colonies. Independent clones of the library used were 5 X 10<sup>6</sup> and actually screened ones were 72.5 X 10<sup>7</sup>, and thus about 5 times were screened. His<sup>+</sup> yeasts in this first screening were 82 clones.

#### 2-4) The second screening; $\beta$ -gal assay

To confirm that in the clones obtained in the first screening, a bait actually bound to a pray to express a gene downstream of the GAL4 promoter, expression of another lac Z gene located downstream of the GAL4 promoter, specifically  $\beta$ -gal activity, was examined. A nylon transfer membrane was placed on the SD/-Trp/-Leu/-His/+3-AT agar plate, and 82 yeast clones which became His<sup>+</sup> in the first screening were cultured and grown on this membrane. Clones having His<sup>+</sup> and the  $\beta$ -gal activity were obtained by measuring the  $\beta$ -gal activity by colony lift filter assay. By this second screening, 74 colonies having the  $\beta$ -gal activity were obtained.

#### 2-5) Sequencing of a pray

Plasmid DNA was harvested from yeast and transferred to *E. coli*, to examine DNA sequences inserted into the clones obtained by screening.

The yeast clones having His<sup>+</sup> and the  $\beta$ -gal activity were scratched from the plates, and cultured on the SD/-Leu medium overnight. Bacterial cells were collected and treated by following the standard method (Kaiser, P. & Auer, B. (1993) Rapid shuttle plasmid preparation from yeast cells by transfer to *E. coli*. Bio Techniques 14: 552) to collect yeast plasmid DNA.

*E. coli* HB101 for electroporation, made competent using HEPES-NaOH was electrotransformed with the plasmid DNA collected from yeast. After electroporation, SOC medium warmed at 37°C was added thereto, and the *E. coli* was cultured with shaking at 37°C for 1 hour to recover. The *E. coli* was spread on the -Leu plate (M9 plate

containing 50 µg/ml ampicillin, 40 µg/ml proline, 1 mM thiamine hydrochloride, -Leu dropout solution) and cultured at 37°C overnight. *E. coli* HB101 has *LeuB* mutation. Therefore, among plasmid DNA obtained from yeast, only library vectors encoding *LEU2* gene which  
 5 can complement *leuB* mutation can transform the *E. coli* HB101 and form colonies on the plate. From grown *E. coli* HB101, plasmid DNA was extracted by the alkaline-SDS method. *E. coli* DH5α was transformed using the harvested plasmid DNA.

Plasmid DNA of pACT2 vector in *E. coli* DH5α was harvested by  
 10 the alkaline SDS method, and purified by the polyethylene glycol precipitation method. Based on Sanger method, the nucleotide sequences of the genes in the vectors were confirmed by the florescent sequencer. In this manner, a novel gene X19 was obtained.

#### 15 2-6) Confirmation by re-transformation

After transforming yeast Y190 with the purified plasmid DNA of pACT2-X19, it was confirmed that sole the fusion protein of the GAL4-AD protein and X19 did not cause transcription from the GAL4 promoter by measuring the β-gal activity. By measuring the β-gal  
 20 activity in Y190 transformed by pASΔNdeI (+2) -XIAP and pACT2-X19, and measuring the β-gal activity in Y190 transformed by pAS-X19 and pACT-XIAP, transcription from the GAL4 promoter, namely, the binding of XIAP and X19 in the yeast nucleus was confirmed.

### 25 3. X19 amino acid sequence homology search

Amino acid sequence homology search was conducted using www service (<http://www.genome.ad.jp>) of Human Genome Analysis Center, Medical Science Institute, The university of Tokyo, and of Supercomputer Laboratory at Institute of Chemistry, Kyoto University  
 30 to predict the functions of X19 from the amino acid sequence.

#### 3-1) Sequence homology search program BLAST

Using the non-redundant amino acid sequence data base nr-aa, sequences homologous to amino acid sequence of X19 were searched  
 35 (blastp search). As a result, X19 was a novel gene having 55% homology to human thioredoxin reductase and 38% homology to human

glutathione reductase. Moreover, functional regions (a redox active center, a FAD-binding region, a NADPH-binding region, a selenocysteine active center) reported in human thioredoxin reductase were completely conserved in the homologous manner in X19 (Figure 1, SEQ ID NO: 1). Therefore, we named X19 human thioredoxin reductase II (TxRII).

#### 4. Obtaining the full length TxRII cDNA

##### 4-1) Obtaining a full length cDNA by colony hybridization

From Human Placenta MATCHMAKER cDNA library, a full length TxRII cDNA was obtained by colony hybridization. For screening, a DNA fragment was amplified by PCR from a partial sequence of the sequenced TxRII and used as a probe.

##### i) Preparation of a membrane for colony hybridization

Human Placenta MATCHMAKER cDNA library was diluted and spread on a LB (ampicillin) plate with a diameter of 150 mm, on which 4 X 10<sup>4</sup> or more colonies can grow per plate. These 12 plates were prepared and cultured at 30°C overnight. The colonies were transferred to a membrane for hybridization, and the membrane for colony hybridization was prepared by following the standard method.

##### ii) Preparation of a probe

About 500 bp DNA fragment at N-terminal side was obtained using the following primers by PCR with the cDNA of TxRII as a template.

TxRII-sF3 5'-TAT GAT CTC CTG GTG GTC-3'

TxRII-sR2 5'-GTC ATC ACT TGT GAT TCC-3'

The amplified DNA fragment was separated by the 1% agarose gel electrophoresis, and purified by the glass matrix method. From the purified DNA fragment, a [<sup>32</sup>P] labeled probe was prepared using the DNA random labeling kit (rediprime DNA labelling system, Amersham) and [ $\alpha$ -<sup>32</sup>P] deoxy-CTP (ICN), and purified by spin column (ProbeQuant G-50 Micro Column, Pharmacia).

##### iii) Hybridization

Hybridization was conducted using a hybridization bottle and

a hybridization oven (TAITEC). The membrane crosslinked with DNA was pre-hybridized in hybridization buffer (10% PEG6000, 1.5% SSPE, 7% SDS) at 65°C for 1 hour. The [<sup>32</sup>P] labeled probe was boiled, immediately cooled, and diluted with hybridization buffer warmed at 65°C and the solution used for prehybridization was replaced by the hybridization buffer. Hybridization was conducted at 65°C overnight.

iv) Washing and autoradiography

Hybridization buffer was washed with washing solution of 0.1xSSC, 0.1% SDS, and the level of washing was appropriately confirmed by a survey meter. Washing solution was replaced several times until a count of washing solution was completely absent, and then the membrane was loaded on the film to detect positive colonies by autoradiography.

v) Isolation of positive colonies

Positive colonies were isolated by a Pasteur pipet, diluted by the different dilution ratios, spread on a LB (ampicillin) plate of 100 mm diameter and cultured at 30°C overnight. Hybridization was conducted by the same manner and single positive colony was obtained. From this, plasmid DNA was harvested and the DNA sequence was determined. SEQ ID NO: 1 shows the nucleotide sequence of TxRII α cDNA determined in this manner.

4-2) Obtaining a full length cDNA by PCR cloning

From Human Placenta MATCHMAKER cDNA library used in the two hybrid system, TxRII gene was attempted to obtain by PCR by combining TxRII specific primers and library vector specific primers. Sequences of used primers were set as follows based on the nucleotide sequences of the clones obtained by colony hybridization.

TxRII specific primer 1

5'-ACA GCT TCT GCC ATC TTC CTC-3'

TxRII specific primer 2

5'-AGA AGG TTC CAC GTA GTC CAC-3'

Library vector specific primer

5'-CCA TAC GAT GTT CCA GAT TAC-3'

PCR was conducted by the combination of TxRII specific primer 1 and the library vector specific primer in the following program, using GeneAmp PCR System 2400 (PERKINELMER).

a) 94°C, 5 min

5 b) 35 cycles of 94°C 30 s, 56°C 30 s, 72°C 1 min and 30 s,

d) 72°C 10 min.

A PCR product was electrophoresed by the 1% agarose gel, excised, and purified to be used as a template for the following PCR. The second PCR was conducted using the combination of the TxRII specific primer 2 and the library vector specific primer by the following program.

a) 94°C, 5 min

~~b) 35 cycles of 94°C 30 s, 56°C 30 s, 72°C 1 min and 30 s,~~

~~d) 72°C 10 min.~~

The PCR product was electrophoresed by the 1% agarose gel, excised, purified, and cloned by using Topo TA cloning Kit (Invitrogen) and DNA sequence of the PCR product was sequenced. As a result, cDNA containing 5'-non amino acid translation region of about 180 bp was obtained and the first methionine (Met) was judged as the first Met due to the presence of Kozak consensus immediately before the methionine. The sequence at N-terminal side, however, was different from that obtained by the yeast two hybrid method. Because the sequence of the second exon and following sequence in this gene was identical to that in TxRII, the gene was decided to be an alternative splicing form of TxRII. The gene obtained by yeast two hybrid method, and the alternative splicing form were designated TxRII $\alpha$  and TxRII $\beta$ , respectively. The second exon and the following part in TxRII $\beta$  is identical to that in TxRII $\alpha$ (SEQ ID NO: 3).

In addition, based on the cDNA nucleotide sequence of TxRIIs, known genomic nucleotide sequences were searched, and the cDNA nucleotide sequence of TxRIIs was mapped on 22q11.2. The genes encoding TxRIIs were present in 70 kbp in this region while separating into 18 exons. The presence of a gene encoding a protein having the binding activity with XIAP or the TxR activity was not predicted in this region.

## 5. Preparation of anti-TxRII antibody

In order to prepare an antibody against human TxRII proteins, a fusion protein with glutathion-S-transferase (GST) protein was purified as an immunogen, and anti-TxRII mouse antiserum was harvested by immunizing a mouse.

### 5-1) Expression of the GST-TxRII $\alpha$ fusion protein

The TxRII $\alpha$  fragment was re-cloned to pGEX vector (Pharmacia) from pACT2-TxRII $\alpha$  to construct pGEX-TxRII $\alpha$ . *E. coli* (DH5 $\alpha$ ) transformed with this pGEX-TxRII $\alpha$  was cultured in a LB-ampicillin medium at 37°C overnight. This cultured medium was added to a fresh LB-ampicillin medium at 100X dilution, and cultured at 37°C. When the turbidity of the culture medium reached about 0.6, IPTG (isopropyl- $\beta$ -D(-)-thiogalactopyranoside) was added thereto at the final concentration of 0.5 mM to express the GST-TxRII $\alpha$  fusion protein, and cultured at 37°C for further 4 hours. The bacterial cells were harvested by centrifugation after the culture.

The collected bacterial cells were well-suspended in ice-cooled PBS containing 1% Tween -20, and completely crushed by ultrasonication. The crushed solution was centrifuged and the supernatant was passed through a GSH-sepharose 4B column (Pharmacia) to adsorb a GST fusion protein on the column. The column was washed well with WE buffer (10 mM  $\beta$ -mercaptoethanol, 2 mM MgCl<sub>2</sub>, 20 mM Tris-HCl, (pH7.5)), and the GST-TxRII $\alpha$  fusion protein was eluted using G buffer (10 mM GSH, 50 mM Tris-HCl, pH 9.6). The eluate was concentrated by 50% glycerol/PBS and the buffer was replaced.

### 5-2) Immunization of the GST-TxRII $\alpha$ fusion protein into a mouse, collecting blood, and confirmation of reactivity

The purified GST-TxRII $\alpha$  fusion protein and Freund's complete adjuvant were emulsified, and intraperitoneally injected into a mouse. This manipulation was repeated once a week for 5 weeks, and blood was collected from the mouse to collect serum containing the anti-TxRII antibody. The immunogen, TxRII overexpressed in mammalian cells, and the reactivity in various cultured cells were confirmed by the Western blotting method using this antiserum.

## 6. Western blotting method (Figure 2)

Soluble proteins were prepared from cultured cells, and protein concentration was measured by following the standard method (M. M. Bradford, Anal. Biochem. 72, 248, 1976), and SDS-PAGE was conducted with 40  $\mu$ g of protein per lane. This was immunodetected with anti-TxRII antiserum and the presence of TxRII protein present in each cultured cell line was confirmed. As a result, the expression of TxRII was confirmed in each type of cultured cells. In Figure 2, TxRII $\alpha$  was the band at around 70 kDa, and TxRII $\beta$  was the band at around 55 kDa. The expression of TxRII $\beta$  was not confirmed in mouse or rat cultured cells. The following 11 cell lines were used as samples.

Raji human Burkitt's lymphoma-derived cell line  
 Jurkat human T cell acute lymphoblastic leukemia-derived cell line  
 HL60 human acute promyelocytic leukemia-derived cell line  
 U937 human histiocytic lymphoma-derived cell line  
 ZR75-1 human epidermic breast cancer-derived cell line  
 HepG 2 human protopathic hepatoblastoma-derived cell line  
 HeLa human uterine cervix cancer-derived cell line  
 A 431 human vulva squamous cell carcinoma-derived cell line  
 MRC-5 human-derived normal fibroblast cell line  
 NIH/3T3 mouse fetus-derived normal fibroblast cell line  
 Rat-1 rat fetus-derived normal fibroblast cell line

## 7. Purification and activity measurement of the recombinant TxRII $\alpha$ protein

### 7-1) Preparation of histidine tag fused TxRII $\alpha$ protein

To pcDNAHis, a mammalian cell expression vector, was sub-cloned a full length TxRII $\alpha$  gene containing 3'UTR (SEQ ID NO: 1). By transfecting this plasmid DNA to a mammalian cells, TxRII $\alpha$  protein in which a histidine tag is added at N-terminal side is overexpressed in the cells. The plasmid DNA was transfected to 293T cells by the lipofection method according to the standard method. The cells were harvested 48 hours after the transfection, and the target protein

was purified by using the kit for purifying a histidine-tag fusion protein.

#### 7-2) Purification of flag-tag fused TxR11 $\alpha$ protein

To pcDNAFlag, a mammalian cell expression vector, the full length gene of TxR11 $\alpha$  containing 3'UTR was sub-cloned. By transfecting this plasmid DNA into mammalian cells, selenocysteine was inserted into a protein, and only a protein in which flag-tag was added at C-terminal side of TxR11 $\alpha$  can be collected with the anti-Flag antibody affinity column.

According to the standard method, using the lipofection method, the plasmid DNA was transfected to 293T cells. The cells were collected 48 hours after the transfection, and the cell extract solution was passed through the anti-Flag antibody affinity column to collect the flag-tag fused TxR11 $\alpha$  protein using a peptide of Flag.

#### 7-3) Purification of the MYC-tag fused TxR11 $\alpha$ protein

To pCMVmyc, a mammalian cell expression vector, the full length gene of TxR11 $\alpha$  containing 3'UTR was sub-cloned. By transfecting this plasmid DNA into mammalian cells, proteins in which MYC-tag is added at N-terminal side in TxR11 $\alpha$  are overexpressed. By following the standard method, using the lipofection method, the plasmid DNA was transfected to 293T cells. The cells were collected 48 hours after the transfection, Protein A sepharose to which the anti-MYC monoclonal antibody was bound was added to the cell extract solution, and gently stirred at 4°C for 2 hours. By centrifuging, the MYC-tag fused TxR11 $\alpha$  protein binding to protein A sepharose to which the anti-MYC monoclonal antibody bound was precipitated, the supernatant was removed, and the proteins were washed several times with NETN buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5% NP-40, 150 mM NaCl).

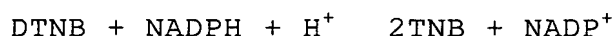
#### 7-4) Activity measurement

By following the standard method (Holmgren, A. and Bjornstedt, M. 1995, [21], Thioredoxin and Thioredoxin Reductase Methods in Enzymol 252: 199), an activity of TxR was measured by the DTNB assay, and the insulin assay.



## i) DTNB assay

DTNB assay is a method in which TNB caused by the TxR activity from DTNB is measured by the absorbance of a thiol at 412 nm based on the following reaction formula. The purified tag fused TxR $\alpha$  protein (1 to 50  $\mu$ l) was added to the assay buffer 1 to mess up to 1.0 ml. The absorbance at 412 nm was measured at 25°C for 5 min (Figure 3).



Assay buffer 1:

100 mM potassium phosphate pH 7.0, 10 mM EDTA, 0.25 mM NADPH, 0.2 mg/ml bovine serum albumin (BSA), 1% ethanol, 1 mM DTNB

As a result, all TxR $\alpha$  purified by three methods was found to have the activity equivalent to that of control TxR derived from *E. coli*. The reason why the activity of histidine and the MYC-tag fused TxR $\alpha$  protein is slightly low is considered that TxR $\alpha$  in which selenocysteine was not incorporated at C-terminal side was mixed to inhibit the reaction.

## ii) Insulin assay

The purified tag-fused TxR $\alpha$  protein (1 to 50  $\mu$ l) was added to the assay buffer 2 and messed up to 1.0 ml. Oxidation of NADPH was measured by decreased absorbance at 340 nm at 30°C for 5 min (Figure 4). The TxR activity reduces Trx and the reduced form Trx further reduces insulin. At this time, the TxR activity can be measured by the amount of NADPH to be oxidized. The amount of oxidized NADPH was calculated by the following calculation formula.

$$\Delta A_{340} \times 0.5 / 6.2$$

Assay buffer 2:

50 mM phosphate buffer pH 7.0, 20 mM EDTA, 80 mM insulin, 0.25 mM NADPH, 16 mM *E. coli* Trx-S2

As a result, all TxR $\alpha$  purified by three methods was found to have the activity equivalent to that of control TxR derived from *E. coli*. The reason why the activity of histidine and the MYC-tag fused TxR $\alpha$  protein is slightly low is considered that TxR $\alpha$  in which selenocysteine was not incorporated at C-terminal side was mixed to inhibit the reaction.

#### 7-5) Inhibitor assay

To compare an enzyme activity of the TxRIIs by the present invention, obtained as a recombinant, and an activity of the natural TxR, an effect of an inhibitor was observed. As an inhibitor for the TxR activity, 1-chloro-2, 4-dinitrobenzene (CDNB) and 13-cis-retinoic acid was used. For confirming the TxR activity, the DTNB assay was used.

The diluted series of the inhibitors was prepared with 0.2 ml of HE buffer (100 mM HEPES buffer pH 7.2, 5 mM EDTA). The tag-fused TxRII $\alpha$  protein was prepared at 3  $\mu$ M and 0.2 ml thereof was added thereto, then 0.2 ml of HE buffer containing 3 mM NADPH and 30 mM DTNB was added thereto. The reaction system of this solution is composed of 100 mM HEPES buffer pH 7.2, 5 mM EDTA, 1  $\mu$ M flag-tag fused TxRII $\alpha$  protein, 1 mM NADPH, and 10 mM DTNB. The amount of reduced insulin was measured by absorbance of a thiol at 412 nm at 25°C for 5min. Figure 5 shows the result.

As a result, the activity of the purified flag-tag fused TxRII $\alpha$  protein was clarified to be effectively inhibited by CDNB and 13-cis retinoic acid, as previously reported in the references of TxR I. The TxRII $\alpha$  of the present invention was predicted to express an enzyme activity by the same mechanism as in the known TxR.

#### Industrial Applicability

Higher animal's TxR was first purified as an enzyme in the 1990's, and the amino acid sequence thereof was reported in 1995. TxR in higher animals was given attention due to the difference in the size and substrate specificity of the proteins from the homologues in lower animals reported previously. The presence of TxRIIs in human, however, was not predicted, and thus the structure and activity of TxRIIs revealed in the present invention is very meaningful. The following is the importance of the present invention in detail.

The present invention provides an important information in screening of anticancer agents. It has been mentioned that TxR is given attention as a target for an anticancer agent. The importance of the present invention is large because it revealed that there are

more than one species of molecules for the target. Specifically, to provide more certain therapeutic effects, an approach for comprehensively controlling the TxR activity including TxRIIs of the present invention is needed. This kind of approach can be possible first by the knowledge of the present invention.

In a cDNA provided by the present invention, there is 3'UTR constituting the stem loop structure essential for translating selenocysteine present close to C-terminus of TxRIIs. This nucleotide sequence supports the expression of the region containing selenocysteine essential for the expression of the TxR activity. The 3'UTR clarified in the present invention is composed of only 130 bp, and the fact that selenocysteine can be translated by such a short sequence is a novel knowledge. Moreover, considering the present invention from the aspect that the XIAP-binding protein was isolated, the protein of the present invention may bind to XIAP serving the control of apoptosis and, thus, may control the functions. The present invention provides a novel technique for promoting apoptosis, through this possibility. Promotion of apoptosis induces the death of abnormal cells, for example, cancer and virus-infected cells, leading to the treatment of the diseases.